Fish cytokines: current research and applications

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Abstract

Cytokines are low molecular weight glycoproteins involved in the regulation of the immune system, and more than 100 cytokines have hitherto been identified in humans. Cytokines similar to those found in humans have also been found in fish. The innate immune response of fish can be examined by using cytokines as markers. We developed a multiplex reverse transcription–polymerase chain reaction assay to analyze the expression of various cytokine genes. Using this assay, we were able to investigate the fish immune response when it had been activated by immunostimulants. The involvement of inflammatory cytokines, such as interleukin-1 β , in the immune system of fish was revealed following administration of an immunostimulant.

Keywords Cytokine · Immunostimulant · Multiplex reverse transcription–polymerase chain reaction assay · Fish

Introduction

A major concern in aquaculture is the occurrence of fish diseases, which have hitherto mainly been controlled by administering antibacterial agents. However, because of the emergence of drug-resistant bacteria and problems associated with food hygiene as a consequence, it is preferable to avoid treating fish diseases with antibacterial drugs (Sakai 1999).

The immune system of higher vertebrates comprises innate and acquired immunity. Acquired immunity involves the recognition of foreign antigens via antigen receptors on T- and B-lymphocyte membranes. These antigen receptors are activated via antigen stimulation and have high affinity to a specific antigen. The establishment of acquired immunity usually requires several days, and a rapid immune response to, for example, microbial infection is not always initiated

(Nakanishi et al. 2018). On the other hand, an innate immune response is required at the early stages of infection and is mainly attained through the action of phagocytes and humoral factors (Nakanishi et al. 2018).

Innate immunity is conventionally regarded as more important than acquired immunity in preventing infectious diseases in fish. Cytokines are mainly secreted by cells of both the innate and acquired immune systems. These molecules effect small physiological changes via specific receptors present on target cell surfaces and are responsible for signal transmission between cells (Zou and Secombes 2016). Cytokines play an important role in the innate immune response of fish. In this review, recent research on cytokine genes is presented, with a specific focus on describing the molecules important to the innate immune responses of fish, and the immunostimulants that elicit them.

Fish cytokines

Cytokines are involved in various regulatory processes in the vertebrate immune system, and over 100 types have been discovered in humans. Recent genomic studies have confirmed that fish have almost all the same cytokines as mammals (Savan and Sakai 2006; Zou and Secombes 2016). In this review, we discuss the main cytokine molecules of fish.

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Interleukin-1 family

Interleukin (IL) 1 is a pro-inflammatory cytokine that plays an important role in innate immunity. In addition to IL-1, around 100 types of IL are presently known in humans. Recent genomic studies have identified IL-1 as the evolutionarily earliest cytokine in fish (Zou et al. 1999; Wang et al. 2009). In mammals, the genes coding for IL-1 α and IL-1 β are found on the same chromosome and are adjacent to one another, whereas in fish, only the gene encoding for IL-1 β has so far been identified. Using DNA vaccination, Kono et al. (2002), reported that IL-1 plays an important role in fish immunity by activating lymphocytes and phagocytic cells and increasing resistance to *Aeromonas hydrophila* infection. Recently, a newer type of *il1b* gene was discovered in Japanese medaka *Oryzias latipes*. This gene is 76.0% homologous to the previously identified *il1b* gene, and is present on different chromosomes (Morimoto et al., personal communication). Furthermore, this gene has been confirmed to have pro-inflammatory actions.

IL-2 family

In the IL-2 family, IL-2, IL-4, IL-7, IL-9, IL-15, and IL-21 are found in humans (Lin and Leonard 2018). IL-2, IL-4/13A, IL-4/13B, IL-7, IL-15, and IL-21 have been reported in fish (Zou and Secombes 2016). As in mammals, the genes of IL-2 and IL-21 in fish are found in tandem (Bird et al. 2005). Two IL-4/13 genes in fish, *il4/13a* and *il4/13b*, have been found to be counterparts of mammalian IL-4 and IL-13, and are thought to have arisen through genome duplication. Moreover, the presence of IL-15 was revealed for the first time in Japanese pufferfish *Takifugu rubripes*, and another *il15* ortholog similar in structure to *il15* has been confirmed in Japanese pufferfish and zebrafish *Danio rerio* (Gunimaladevi et al. 2007).

IL-6 family

The molecules identified in humans which belong to the IL-6 cytokine family are IL-6, IL-11, leukemia inhibitory factor (LIF), oncostatin M (OSM), and ciliary neurotrophic factor (CNTF). The IL-6 sub-family cytokines are major players in hematopoiesis, and have pro- and anti-inflammatory properties. IL-6, IL-11 and CNTF-like occur in fish, and M17 has been identified as a fish-specific molecule that could possibly be an ancestral molecule to LIF and OSM (Hanington and Belosevic 2007). Although the functions of these molecules have not been clarified, recombinant IL-6 has been reported to promote phagocyte proliferation in rainbow trout (Costa et al. 2011). The rIL-6-induced transient

expression (up to 4 h post-stimulation) of SOCS1-3 and interferon (IFN) regulatory factor (IRF)-1, and more sustained upregulation of antimicrobial peptide (AMP) gene expression at a similar level, were found in primary (head kidney derived) macrophage cultures (Costa et al. 2011).

IL-10 family

IL-10, IL-19, IL-20, IL-22, IL-24, IL-26, and IFN- γ are cytokines of the IL-10 family. In general, fish IL-10, similarly to mammalian orthologs, acts as a suppressor and exerts a conserved role in dampening inflammatory responses. The functions of IL-10 have been characterized in goldfish and carp (Piazzon et al. 2015). In fish, *il10* and *il19/20* genes are present in tandem on the same chromosome and code for IL-22, IL-24, and IFN- γ (Savan and Sakai 2006). We discovered that a newly identified IFN- γ gene (*ifngrel*) exists next to the *ifng* gene (Igawa et al. 2006). IFN- γ rel is thought to be involved in immune defense against bacterial infection. Elevated expression of the *ifngrel* gene in the carp, rohu *Labeo rohita*, has been reported following infection of the fish with *A. hydrophila*, *Edwardsiella tarda*, or *Shigella flexneri* (Swain et al. 2015).

IL-17 family

Genes of six members of the IL-17 family, IL-17A, IL-17B, IL-17C, IL-17D, IL-17E, and IL-17F, exist in humans. IL-17A/F1, IL-17A/F2, IL-17A/F3, IL-17C, IL-17D, and a fish-specific IL-17 N, have been reported in fish (Korenaga et al. 2010). We examined the function of Japanese pufferfish IL-17A/F1 using its recombinant (r) protein, and found that it enhanced the expression of tumor necrosis factor - α , (*tnfa*), *il1b*, and *il6* and activated phagocytes (Kono et al. 2011; Takahashi et al. 2020). In yellow croaker, IL-17A/F1 has a higher ability to activate the transcriptional activity of NF- κ B than IL-17A/F2 and IL-17A/F3 (Ding et al. 2016). In rainbow trout, rIL-17A/F2 is able to induce IL-6, chemokine CXCL-8 and antimicrobial peptide β -defensin expression in splenocytes (Monte et al. 2013). Grass carp rIL-17A/F1 also induces several pro-inflammatory cytokines including *tnfa*, *il1b*, *il6* and the chemokine *cxc18* in head kidney leukocytes (Du et al. 2015). Furthermore, the gene expression of digestive enzymes and antibacterial substances is suppressed when the *il17a/f1* gene is knocked out by the clustered regularly interspaced short palindromic repeats (CRISPR)/CRISPR-associated protein (Cas) 9 system in Japanese medaka *O. latipes* (Okamura et al. 2020), suggesting that medaka IL-17A/F1 may have a role in maintaining healthy intestines by modulating the gut microbiome via transcriptional control of the affected genes.

Chemokine family

Chemokines are produced in large quantities in areas of inflammation and induce the migration of leukocytes from blood vessels to inflamed tissues. To date, more than 50 chemokines have been identified in humans. Chemokines have four highly conserved cysteine residues and are classified into four subfamilies, namely CXC, CC, CX3C, and XC, according to the motif formed by the two N-terminal cysteine residues. Many chemokines have also been identified in fish. More than 100 types of zebrafish chemokines have been reported, although none that belong to the CX3C subfamily (Nomiya et al. 2008). Several CXC chemokines, including CXCL8_L1 (IL-8), CXCL11_L1 (γ IFN-inducible protein), CXCL_F4 and CXCL_F5 were found to increase in rainbow trout cell lines (i.e., RTG-2 and RTS-11 cells) after rIL-1 β stimulation (Chen et al. 2013).

Interferon family

Interferons are types of cytokines produced by animal cells in response to the invasion of viruses, heterologous RNA, and certain sugars. Cells infected with viruses are known to exhibit an interference phenomenon that blocks infection by other viruses. Human IFNs include type I IFNs (13 types of IFN- α , IFN- β , IFN- ω , IFN- ϵ , and IFN- κ), type II IFN (IFN- γ), and type III IFNs (IFN- λ 1, IFN- λ 2, and IFN- λ 3). In fish, type I IFNs (homologs to the human IFN- α and IFN- β) exhibit antiviral activity, and type II IFN (IFN- γ) have also been reported to exhibit bactericidal activity against intracellular parasitic bacteria (Zou and Secombes 2016). A type I IFN in fish has been shown to have antiviral activity, but the gene encoding it has introns and exons, unlike mammalian genes coding for IFN (Zou and Secombes 2016).

TNF family

The TNF family includes proteins with the following motifs: (LV)-x-(LIVH)-x3-G-(LIVMF)-Y-(LIYMHY)2-X2-(QEKHL)-(LIVMGT)-x-(LIVMFY). More than 20 TNF molecules have been reported in humans, including TNF- α , lymphotoxin (LT)- α cytokines involved in apoptosis, the Fas ligand, the TNF-related apoptosis-inducing ligand (TRAIL), the receptor activator of nuclear factor- κ B (RANK) ligand, the TNF receptor superfamily member 4 (OX40) ligand, and proliferation-inducing ligand 20 (TNF ligand superfamily member 13; APRIL 20). At least ten TNF genes have been identified in pufferfish (Biswas et al. 2015). TNF- α is the most immunologically important molecule in the TNF family. In humans, three genes, *tnfa*, *tnfb* (also called *lta*), and *ltb* exist in tandem. In fish, two genes, *tnfa* and *tnfn* are duplicated in tandem on the chromosome (Savan et al. 2005). In addition, unlike mammals, fish have

another *tnfa* on another chromosome (Kinoshita et al. 2014). It has been suggested that members of the fish TNF- α family are involved in the regulation of leukocyte homing, proliferation and migration. Constitutive expression of TNF- α was detected in the trout thymus, which suggests that it could play a role in promoting thymocyte growth (Hino et al. 2006). Members of the TNF- α family in fish exert pro-apoptotic activity, as do their mammalian homologues. High doses (> 400 ng/mL) of Chinese perch TNF- α induced apoptosis in human Hela cells (Xiao et al. 2007). In tilapia, TNF- α has been shown to upregulate granzyme expression in non-specific cytotoxic cells and to protect these cells from activation-induced cell death (Praveen et al. 2006).

Other cytokines

Other important cytokines in fish include the IL-12 subfamily molecules, IL-18, transforming growth factor (TGF)- β , and macrophage colony-stimulating factor (M-CSF) (Zou and Secombes 2016). The function of fish IL-12 has been studied in two different paralogues of the p40 chain heterodimerized with a common p35. In grouper, rIL-12 increased the concanavalin A (ConA)-induced proliferation of peripheral blood lymphocytes and *tnfa* expression (Tsai et al. 2014). In amberjack *Seriola dumerili*, rIL-12-mixed *Nocardia seriolae*-FKC vaccine induced cell-mediated immunity and production of type 1 helper (Th1) cells with antigen memory against *N. seriolae* infection (Matsumoto et al. 2017). IL-18, as well as IL-1 β , is released after cleavage by caspase-1 following stimulation of inflammasome with pathogen-associated molecular patterns or damage-associated molecular patterns. Unlike IL-1 β , which is transiently induced by inflammatory stimuli, IL-18 is constitutively expressed in tissues of the innate and adaptive immune defense systems in fish (Zou et al. 2004). Most studies on TGF- β in fish have focused on its function, in combination with other cytokines, as a driver of Th17 differentiation (e.g., IL-6), but it is also a key immunosuppressive cytokine secreted by regulatory T cells (Haddad et al. 2008; Wei et al. 2015). Fish M-CSF was first identified from goldfish (Barreda et al. 2005), and was shown to induce the differentiation of monocytes into macrophages and the proliferation of monocyte-like cells with a mononuclear phagocyte function (Grayfer et al. 2009).

Immune function enhanced by immunostimulants

Many immunostimulants have been developed for use in aquaculture, the major ones of which are shown in Table 1 (Sakai 1999). Immunostimulants are generally considered to prevent disease by increasing the innate immune response;

Table 1 Major immunostimulants influencing fish immune response

Immunostimulants	References
Synthetic chemicals	
Levamisole	Kajita et al. 1990
FK-565	Kitao and Yoshida 1986
MDP	Kodama et al. 1993
Imiquimod	Kitao et al. 2009
Biological substances	
Bacterial derivatives	
FCA	Olivier et al. 1985
Peptidoglycan	Itami et al. 1996
EF203	Yoshida et al. 1993
LPS	Salati et al. 1987
Yeast glucan	Dalmo and Bogward 2008
Polysaccharides	
Chitin	Sakai et al. 1992
Chitosan	Siwicki et al. 1994
Lentinan	Yano et al. 1989
Schizophyllan	Itami et al. 1994
Oligosaccharide	Yoshida et al. 1995
Animal extracts	
Ete (tunicate)	Davis and Hayasaka 1984
Hde (abalone)	Sakai et al. 1991
Firefly squid	Siwicki et al. 1996
Plant extracts	
<i>Quillaja</i> saponin	Ninomiya et al. 1995
Hops	Galina et al. 2009
Vaccines (inducing natural immune responses)	
Vibrio	Sakai et al. 1995a, b
Aquabirnavirus	Yamashita et al. 2009
Nutritional factors	
Vitamin C	Pohlenz and Gatlin 2014
Vitamin E	Pohlenz and Gatlin 2014
Hormones, cytokines and others	
Lactoferrin	Sakai et al. 1993
Lysozyme	Siwicki et al. 1998
Growth hormone	Sakai et al. 1996
Prolactin	Sakai et al. 1996
Cytokines (recombinant protein)	
Interleukin-1 β	Kono et al. 2002
Chemokine	Kono et al. 2003
Type-I interferon	Robertsen et al. 2003
Interferon- γ	Jung et al. 2012
Nucleic acid	
Nucleotides	Sakai et al. 2001
CpG motif of DNA	Tassakka and Sakai 2004

MDP Muramyl dipeptide, FCA Freund's complete adjuvant, LPS lipopolysaccharide

we describe here the immune mechanisms that they activate in fish.

Activation of phagocytes

Phagocytic cells are functionally activated by the administration of immunostimulants. The functions of phagocytic cells can be classified as migratory, phagocytic, and bactericidal, with phagocytosis the one most intensively investigated in immunostimulation research. Generally, the phagocytosis rate and phagocytic index of the renal and intraperitoneal phagocytes of fish treated with immunostimulants, e.g., levamisole (Kajita et al. 1990), and lactoferrin (Sakai et al. 1993), significantly increases. Furthermore, the phagocytosis rate and phagocytic index increased significantly after phagocytes had been cultured in vitro in a medium containing an immunostimulant (Sakai et al. 1995a, b).

The sterilizing pathways of phagocytic cells can be broadly divided into two types: systems that depend mainly on oxygen and those that do not. Oxygen-dependent systems basically comprise oxygen compounds (e.g., O₂ and H₂O₂) and nitrogen compounds (e.g., NO, NO₂, N₂O₃, and NO₂⁺). Bactericidal substances, such as lysozyme and defensin, are important oxygen-independent systems. The administration of chitin, lactoferrin, or levamisole as immunostimulants enhanced the bactericidal action of oxygen compounds and oxygenated compounds (superoxides) produced by phagocytes (Kajita et al. 1990; Sakai 1999; Sakai et al. 1992). The migration ability of phagocytic cells was reported to significantly increase in channel catfish fed with yeast glucan-supplemented feed (Duncan and Klesius 1996).

Activation of lymphocytes

Lymphocytes have also been reported to be activated by the administration of immunostimulants. A lymphocyte mitogenic response was promoted in rainbow trout (*Oncorhynchus mykiss*) leukocytes cultured in a culture medium [with ConA and lipopolysaccharide (LPS)] containing firefly squid (*Watasenia scintillans*) extract in vitro; the production of macrophage-activating factor increased significantly (Siwicki et al. 1996). Similar results have been obtained with a growth hormone and glycyrrhizin (Jang et al. 1995; Sakai et al. 1996).

Enhancement of complement activation

The complement system is a series of enzyme systems that can destroy foreign substances either alone or in coordination with antibodies. The activation of complement systems can enhance the phagocytic ability of phagocytes, and immunostimulants can increase this activation. Engstad et al. (1992) reported an increase in a complement factor

in Atlantic salmon (*Salmo salar*) injected with yeast glucan. Similar results have been reported in channel catfish (*Ictalurus punctatus*) (Li and Lovell 1985) and Atlantic salmon (Hardie et al. 1991) treated with high doses of vitamin C, and rainbow trout (Kajita et al. 1990) treated with levamisole.

Activation of lysozyme

Lysozyme is a heat-resistant antibacterial enzyme found in the serum and body surface mucus of fish. Glucans are generally known to increase lysozyme activity (Engstad et al. 1992). However, lysozyme activity did not increase in fish in response to the administration of chitin (Sakai et al. 1992) or lactoferrin (Sakai et al. 1995a, b).

Fish have two types of lysozyme genes: chicken-type (C-type) and goose-type (G-type) (Callewaert and Michiels 2010; Hikima et al. 2002). Lytic activity of fish lysozyme has been detected in skin mucus, serum, the kidneys (including head and trunk kidneys), liver, gills, and eggs (Saurabh and Sahoo 2008; Yano 1995). The expression of the lysozyme gene in these tissues was shown by Callewaert and Michiels (2010) and Hikima et al. (2002). The messenger RNA levels of C-type and G-type lysozymes are upregulated in many fish species following challenge with immunostimulants and pathogenic microbes (Hikima et al. 1997; Jiménez-Cantizano et al. 2008; Ye et al. 2010). In turbot *Scophthalmus maximus*, two G-type lysozyme genes have been identified, one of which showed significant upregulation in the intestine following infection with *Vibrio anguillarum* and *Streptococcus iniae* (Gao et al. 2016). Furthermore, recombinant fish C-type lysozymes possess bacteriolytic and/or bactericidal activities against several Gram-positive and Gram-negative bacterial pathogens (Minagawa et al. 2001; Hikima et al. 2001; Ye et al. 2010; Gao et al. 2012).

Disease prevention with immunostimulants

Diseases that can be prevented by the use of immunostimulants include streptococcosis, vibriosis, and furunculosis, all of which are caused by extracellular bacteria. Few cases of immunostimulants being effective against viral diseases or intracellular bacteria, such as bacteria responsible for bacterial kidney disease, have been documented. In most cases, activated phagocytes are effective against extracellular bacteria. The activation of IL-1 β and TNF- α is indispensable for this because their activation effects several innate immune responses including phagocytosis (Sakai 1999). The activation of type-I IFN is most effective against viral infections. In Japanese flounder *Paralichthys olivaceus*, rIFN- γ is effective in reinforcing immune responses and preventing edwardsiellosis (Jung et al. 2012).

Expression of cytokine genes in immunostimulant-treated or bacteria-infected fish assayed by multiplex reverse transcription–polymerase chain reaction assay

Multiplex reverse transcription–polymerase chain reaction assay

The multiplex reverse transcription–polymerase chain reaction (RT-PCR) assay is designed to simultaneously amplify multiple genes during one test in one reaction tube (Bonetta 2006). We performed multiplex RT-PCR assay using chimeric primers for each gene designed by GenomeLab GeXP (Beckman Coulter), and quantified each gene. As the level of gene expression is investigated by amplifying and analyzing multiple genes in one tube with this method, both the duration and cost of analysis could be greatly reduced, enabling comprehensive examination of the expression levels of various genes.

In this multiplex RT-PCR reaction, each chimeric primer (i.e., a primer composed of both a gene-specific and universal sequences) was constructed so that the PCR products had different sizes. The PCR products of each gene were synthesized by using these chimeric primers so that universal sequences were added to both the 5' and 3' ends during the reverse transcription reaction and several PCR cycles. Thereafter, a universal primer labeled with a fluorescent dye was used during synthesis, so that a fluorescent PCR product was produced. The PCR products were fractionated based on size via capillary gel electrophoresis, and the gene expression level was calculated by detecting the fluorescence intensity of each gene.

Development of a multiplex RT-PCR assay to quantify fish cytokine genes

We successfully developed a multiplex RT-PCR assay for the comprehensive quantification of fish cytokine gene expression. We clarified the existence of, and cloned, various cytokine genes from the Japanese pufferfish *T. rubripes* (Table 2) (Kono et al. 2013).

Expression of cytokine genes in immunostimulant-treated or bacteria-infected fish

The effectiveness of fish immunostimulants was examined using the above-mentioned cytokine-multiplex RT-PCR assay. Table 3 shows the changes over time in the expression of various cytokine genes, with LPS causing a strong

Table 2 Cytokine gene expression examined in puffer fish by using a cytokine-multiplex reverse transcription–polymerase chain reaction assay

Cytokines	Abbreviation (gene)	Inflammation	Anti-inflammation	T-cell proliferation/differentiation	B-cell activation/differentiation	NK cell stimulation	Anti-viral activity
Interleukin-1 β	IL-1 β (<i>il1b</i>)	●					
Interleukin-2	IL-2 (<i>il2</i>)			●			
Interleukin-4/13A	IL-4/13A (<i>il4/13a</i>)		●		●		
Interleukin-4/13B	IL-4/13B (<i>il4/13b</i>)		●		●		
Interleukin-6	IL-6 (<i>il6</i>)	●			●		
Interleukin-7	IL-7 (<i>il7</i>)				●		
Interleukin-10	IL-10 (<i>il10</i>)		●				
Interleukin-15	IL-15 (<i>il15</i>)			●			
Interleukin-17A/F3	IL-17A/F3 (<i>il17a/f3</i>)	●					
Interleukin-18	IL-18 (<i>il18</i>)	●					
Interleukin-12p35	IL-12p35 (<i>il12p35</i>)					●	
Interleukin-12p40	IL-12p40 (<i>il12p40</i>)					●	
Interleukin-21	IL-21 (<i>il21</i>)			●			
Interferon- γ	IFN- γ (<i>ifnγ</i>)						●
Type I-interferon 1	Type-I IFN (<i>ifn1</i>)						●
TNF- α	TNF- α (<i>tnfa</i>)	●					

NK Natural killer

Table 3 Cytokine responses in pufferfish anterior kidney cells treated with LPS or polyinosinic–polycytidylic acid [poly(I:C)]

Cytokines	LPS			Poly(I:C)		
	1 h	12 h	24 h	1 h	12 h	24 h
IL-1 β	↑↑	↑↑↑	↑↑	↑↑	↑↑	↑
IL-2	→	→	↑	→	→	↑
IL-4/13A	→	↓	↓	↓	→	↓
IL-4/13B	→	→	→	→	→	→
IL-6	↑↑↑	↑↑↑	↑↑	↑↑↑	↑↑	↑↑
IL-7	→	↓	→	→	→	→
IL-10	↑	→	↑↑	→	→	→
IL-12p35	↑↑	↑	→	↑↑	↑↑	→
IL-12p40	↑	↑	↑	↑↑	↑↑	↑
IL-15	→	→	→	→	→	→
IL-17A/F3	↑↑	→	↓	→	→	→
IL-18	→	→	→	→	→	→
IL-21	→	→	→	↓	→	↑
IFN- γ	↑↑	↑↑	→	↑↑	↑↑	↑↑
Type-I IFN	ND	ND	ND	↑	↑↑↑	↑
TNF- α	↑	↑	↑	↑	↑↑↑	↓

ND Not determined; for other abbreviations, see Tables 1 and 2

inflammatory reaction and polyinosinic–polycytidylic acid [poly(I:C)] exhibiting antiviral activity. In pufferfish treated with LPS, the expression of IL-1 β , TNF- α , IL-6, and IL-17A genes as pro-inflammatory cytokines was confirmed to increase 4–8 h after administration. Increased expression of IL-4/13A and IL-12p35 genes was also observed. At 24 h after LPS administration, the expression of the IL-10 gene

increased, whereas that of IL-1 β and TNF- α genes decreased to pre-stimulation levels. An increase in the expression of type-I IFN genes was not confirmed. In cells treated with poly(I:C), an increased expression of IL-6, TNF- α , and other genes was observed, similar to that for LPS administration, but with a marked increase in type-I IFN gene expression. Thus, LPS and poly(I:C) activated different cytokines.

Fig. 1 Multiplex reverse transcription–polymerase chain reaction assay for messenger RNA expression of cytokines in fugu *Takifugu rubripes* exposed to *Edwardsiella tarda* (OA-3). The heat map depicts the relative expression level in the spleen compared to that in the uninfected control. The color scale illustrates the relative expression level of the cytokines: high relative expression (red), low relative expression (green). IL Interleukin, TGF transforming growth factor, TNF-N tumor necrosis factor-N, CSF colony-stimulating factor, I-IFN-I type 1 interferon

The cytokine responses in pufferfish infected with *E. tarda* are shown in Fig. 1. Increased IL-21 expression was observed on the first day after infection, and TNF- α expression levels were increased on the fifth day. In contrast, the expression of IL-1 β , IL-10, IL-17A, type-I IFN, and IL-4/13A genes decreased 7 days after infection.

Conclusion

In this review, we have described the roles of cytokines and their use as indices of innate immunity in fish, and introduced methods that can be used to detect them (e.g., the multiplex RT-PCR assay). However, research to elucidate the functions of cytokines in fish has only recently begun, and further work is necessary for the selection of, for example, appropriate immunostimulants and vaccine adjuvants for the prevention of infections in farmed fish.

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